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Abstract: Gravity-driven membrane (GDM) filtration is a promising tool for low-cost decentralized drinking water production. The biofilms in GDM systems are able of removing harmful chemical components, particularly toxic cyanobacterial metabolites such as microcystins (MCs). This is relevant for the application of GDM filtration because anthropogenic nutrient input and climate change have led to an increase of toxic cyanobacterial blooms. However, removal of MCs in newly developing GDM biofilms is only established after a prolonged period of time. Since cyanobacterial blooms are transient phenomena, it is important to understand MC removal in mature biofilms with or without prior toxin exposure. In this study, the microbial community composition of GDM biofilms was investigated in systems fed with water from a lake with periodic blooms of MC-producing cyanobacteria. Two out of three experimental treatments were supplemented with dead biomass of a MC-containing cyanobacterial strain, or of a non-toxic mutant, respectively. Analysis of bacterial rRNA genes revealed that both biomass-amended treatments were significantly more similar to each other than to a non-supplemented control. Therefore, it was hypothesized that biofilms could potentially be 'primed' for rapid MC removal by prior addition of non-toxic biomass. A subsequent experiment showed that MC removal developed significantly faster in mature biofilms that were pre-fed with biomass from the mutant strain than in unamended controls, indicating that MC degradation was a facultative trait of bacterial populations in GDM biofilms. The significant enrichment of bacteria related to both aerobic and anaerobic MC degraders suggested that this process might have occurred in parallel in different microniches.

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Priming of microbial microcystin degradation in biomass-fed gravity driven membrane filtration biofilms

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Abstract

Gravity Driven Membrane (GDM) Filtration is a promising tool for low-cost decentralized drinking water production. The biofilms on GDM systems are able to remove harmful chemical components, in particular toxic cyanobacterial metabolites such as microcystins (MCs). This is relevant for the application of GDM filtration, because anthropogenic nutrient input and climate change have led to an increase of toxic cyanobacterial blooms. However, removal of MCs in newly developing GDM biofilms is only established after a prolonged period of time. Since cyanobacterial blooms are transient phenomena, it is important to understand MC removal in mature biofilms with or without prior toxin exposure. We studied microbial community composition of GDM biofilms in systems fed with water from a lake with periodic blooms of MC-producing cyanobacteria. Two of three experimental treatments were supplemented with dead biomass of a MC-containing cyanobacterial strain, or of a non-toxic mutant, respectively. Analysis of bacterial rRNA genes revealed that both biomass-amended treatments were significantly more similar to each other than to an unamended control. We thus hypothesized that biofilms could be potentially 'primed' for rapid MC removal by prior addition of non-toxic biomass. A subsequent experiment showed that MC removal developed significantly faster in mature biofilms pre-fed with biomass from the mutant strain than in unamended controls. Therefore, MC degradation was a facultative trait of bacterial populations in GDM biofilms. The significant enrichment of bacteria related to both, aerobic and anaerobic MC degraders suggested that this process might have occurred in parallel in different microniches.

Key words: Gravity Driven Membrane Filtration; biofilms; cyanotoxins; microbial community structure

Introduction

Harmful algal blooms (HABs) have become a global concern in many freshwater, estuarine and marine ecosystems [56]. Triggers for the rise of HABs are an interplay of climate change related factors, i.e. increasing water temperature, or changes in water body stratification patterns, in combination with anthropogenic eutrophication [62]. Frequently, the dominant HAB-forming species are related to various genera of cyanobacteria, such as *Microcystis, Nodularia, Anabaena*, *Trichodesmium* and *Planktothri*x [56], which are all known producers of toxic secondary metabolites. Microcystins (MCs) are amongst the most common toxins produced by cyanobacteria in fresh and brackish water systems [70]. These cyclic peptides are classified as potent hepatotoxins; they are taken up by hepatocytes where they inhibit the activity of eukaryotic protein phosphatase 1 and 2A [27]. Depending on the concentration and exposure time MCs can cause acute [2] or chronic [25] poisoning. Exposure to sublethal MC concentrations induces hepatic oxidative stress [30], and some studies indicate that MC promote liver tumor [37]. MC concentrations can rise up to 1800 μg L^{-1} during the collapse of HABs [70].

A number of bacteria have been described as MC degraders, e.g., *Sphingomonas* sp. [38], *Stenotrophomonas* sp. [10], *P. aeruginosa* [75], *Microbacterium* sp. [64] and *Burkholderia* sp. [46]. While the details of the MC degradation pathway are not clear, several studies suggest that degradation might be carried out by intracellular enzymes of the *mlr* gene cluster [6][38]. Other studies indicate that other degradation pathways might exist [50][23].

Drinking water is the main source of MC uptake for humans and animals via oral ingestion [25]. WHO (World Health Organization) has proposed an upper limit of 1 μg L^{-1} of a common MC variant (MC-LR) in safe drinking water [81]. Conventional water treatment methods such as coagulation are effective to remove intact cyanobacterial cells but not the toxins that are dissolved or present in the fragments of broken cells [12]. Powdered activated carbon [13], slow sand filtration [28] reverse osmosis [52] and filtration membranes [26] are all potential means for MC removal. Chlorination [33] and ozonation [66] have also been suggested most effective for this purpose. Various combinations of these methods are commonly used in developed countries to provide safe drinking water free of either cyanobacterial biomass or free cyanotoxins.

Community-scale processing technology may not always be available in developing and transient countries to treat surface waters during HABs. Gravity-driven membrane (GDM) filtration systems represent a decentralized alternative to produce drinking water at a small scale. In contrast to other membrane-based water purification approaches, biofilm formation is tolerated in the GDM system, and no pre- and/or post-treatment, chemical cleaning, backwashing or flushing is used. The

GDM filtration setup is simple; it operates under ultra-low pressure in a dead-end mode, and GDM systems have been shown to operate stable over several months [60]. GDM filtration is a cost-effective technology to remove suspended organic and inorganic solids, pathogens and other diseases vectors from the raw water [60]. While the undisturbed biofilm formation is one of the main causes for initial flux decline in GDM systems, an equilibrium is eventually reached where the flux remains stable at a lower level over extended periods of time [17].

The biofilm of GDM filtration systems can improve permeate quality [11], e.g., it may delay microbial regrowth via the reduction of readily available organic carbon [16]. Moreover, biofilms were shown to be effective also against cyanotoxins: MCs in concentrations typically observed during HABs can be completely removed after an adaptation period of the biofilms of approximately 2 weeks [43]. Differences between the phylogenetic composition of bacteria in MC-treated biofilms and untreated controls suggest that *Betaproteobacteria* might be particularly relevant for MC degradation. Other reports also point to bacteria from this class (*Burkholderia* sp. and *Methylobacillus* sp.) as potential MC degraders [46][36].

The communities described by Kohler et al. were conspicuously similar in experimental treatments where MC-containing cyanobacteria were either added as live cells or as broken cell fragments [43]. Thus, it is not clear if the MC-degradation capacity of these biofilms was indeed triggered by the presence of the toxin itself, or if the growth of facultative MC-degrading bacteria was favoured by the richer conditions due to the addition of biomass. This difference might be of practical importance in the long-term use of GDM filtration systems. Specifically, it is conceivable that such systems might develop a potential for rapid toxin degradation even in the absence of HABs, e.g., triggered by prior input of biomass from non-toxic algal species.

The aim of this study was to explore the hypothesis that GDM biofilms would develop highly similar community composition when fed with biomass from either a toxic or a non-toxic strain of a cyanobacterial species (*Microcystis* sp.). In addition, we wanted to test if mature, biomass-amended biofilms with no prior exposure to MCs would exhibit a more rapid toxin degradation response than biofilms that had matured without biomass addition.

Materials and Methods

Cyanobacterial cultures

Microcystis aeruginosa PCC 7806 (wild type) and a non microcystin producing mutant strain [19], were grown in cyanobacterial growth medium [41] at 20 °C under constant light at 3.5 µmol quanta m^{-2} s^{-1}. Several Erlenmeyer flasks were inoculated with the wild type and the mutant strain to produce enough

biomass for the experiments. The cultures were grown for approximately 3 weeks. After this period the cultures were harvested, portioned into 50 mL tubes and stored frozen at -20 °C. For the experiments, these tubes were subjected to 3 freeze-thaw cycles in order to lyse the cyanobacterial cells, and to release the microcystins (MCs) from the wild type strain. The absence of MCs in identically processed samples of the mutant strain was confirmed by HPLC-MS analysis (see below).

Experimental system

Water from Lake Zurich from 5 m depth was pumped into the gravity-driven membrane (GDM) filtration system by a centrifugal pump. The water passed through three sedimentation tanks and was collected into a 40 L feed water tank (Figure 1). The feed water was distributed to the biofouling monitors by silicon tubes. Between the feed water tank and the biofouling monitors there was a syringe connection to spike substrates into the system without direct contact with feed water. A polyethersulfone ultrafiltration membrane with 150 kDa nominal cut-off (PBHK, Biomax Millipore, Billerica, MA, USA) was placed in each biofouling monitor. The biofouling monitors, the permeate collection bottles and their connections were all sterilized. The membrane was first soaked in 40 % ethanol for 1.5 h and then placed in sterile deionized water overnight prior to the experiments [31]. The GDM system was operated under ultra-low pressure (65 mbar). The whole system except for the permeate collection bottles was kept in the dark and it was operated at room temperature.

Experimental procedure

Two experiments with comparable setup were conducted, the first one in June 2014 and the second in March 2016. In experiment A (Exp A) three GDM filtration variants were set up in parallel: the unamended treatment only received lake water (LW); the wildtype treatment (WT) was additionally supplied with MC-containing biomass from *M. aeruginosa* and the mutant treatment (MUT) was supplied with the same amount of MC-free biomass from the mutant strain of *M. aeruginosa*. In experiment B (Exp B) the same three treatment types as in Exp A were set up initially in triplicates. However, once the WT treatment had reached 100 % of MC removal efficiency, we changed the conditions in the MUT and LW treatments by supplying them with the same amount of MC-containing biomass from *M. aeruginosa* that was added to the WT treatment (and discontinuing the addition of biomass from the mutant strain in MUT treatment). In both experiments the corresponding type of biomass was supplied every 24 h. No biomass was supplied and no data were collected in Exp B during days 21 and 22. The amount of dry cell weight supplemented to WT and MUT was chosen to roughly mimic an intense natural bloom situation, i.e., on average 15 mg day^{-1}, with no significant

differences between the treatments and experiments. However, the microcystin content of this biomass differed between the experiments; it was 18 µg microcystin L^{-1} day^{-1}, and 92 µg L^{-1} day^{-1} in Exp A and B. respectively. This differences in MC biomass quota were likely related to the growth phase of cyanobacterial cells at the time point of harvesting [79]. The duration of Exp A and B was 20 days and 39 days, respectively. The permeate flux was monitored every 24 h, and a sample for the quantification of MC concentrations was collected daily from the permeate. The transmembrane flux was calculated from the volume collected daily in the permeate collection bottles and the membrane area (0.00191 m^2). The biofilm thickness and structure was assessed at the end of the experiments using optical coherence tomography (OCT) (model 930 nm Spectral Domain, Thorlabs GmbH, Dachau, Germany). The mean thickness was determined by Matlab® (MathWorks, Natick, US) according to Derlon et al. [15]. The MC removal efficiency was calculated from the difference between the amount of MC added and the amount of toxin measured in the permeate collection bottles.

Microcystin quantification

Due to a change in instrumentation, the quantification of MCs in the two experiments was performed with different protocols and equipment. In Exp A MCs were extracted from the total collected permeates by solid phase in a C18 cartridge (1 g, 60 mL, Mega Bond Elute, Varian, Agilent Technologies, Switzerland). Next, MCs were eluted in 100 % methanol, dried in a vacuum rotary evaporator at 40 °C and 35 mbar, re-dissolved in 1 mL 60 % methanol, filtered with a polyethersulphone syringe filter 0.2 µm (IC Acrodisc, Pall Corporation) and quantified as described in Kohler et al. using high-performance liquid chromatography (HPLC) on a Shimadzu 10 AVP system equipped with a photodiode array detector and a C18 Hydrosphere column (YMC, 4.6 × 250 mm, Stagroma, Switzerland) [43]. Two microcystins from *M. aeruginosa* PCC7806, [D-Asp^3] MC-LR (*m/z* 981.5) and MC-LR (*m/z* 995.5) were extracted and purified (99 %) through HPLC and their specific molar absorption coefficient was used for preparing standard solutions to prepare a calibration curve. Quantification of total MC concentration was achieved by determining the summed peak areas of each compound recorded at 239 nm wavelength.

In Exp B 1 mL of sample was collected from the permeate collection bottles, filtered with a polyethersulphone syringe filter 0.2 µm (IC Acrodisc, Pall Corporation) and mixed with methanol achieving a methanol concentration of 70 %. MCs were directly analysed on a HPLC system (1260 Infinity series, Agilent Technologies) and detected on an API 5000 triple quadrupole mass spectrometer (AB Sciex). The toxins were separated in a Synergi Fusion-RP C18 column (50 x 2.00 mm, 4 µm particle size, Phenomenex), the column temperature was constant at 60 °C. Solvent A was 1 mM Formic Acid

(Fluka) in water and solvent B was 100 % methanol (J.T. Baker, Avantor). The flow rate was kept at 1 mL min^{-1}. The following gradient was applied: Solvent B from 20 % to 80 % in 5 min; B 100 % for 2.5 min and B 20 % for 2.5 min. Nodularin (5 µg L^{-1}) was used as the internal standard. The concentrations of MCs and nodularin (*m/z* 825.5) for the calibration curve and internal standards respectively, were determined as described above. The software Analyst (version 1.6.1, AB Sciex) was used for the data acquisition and the MC quantification was done using the MultiQuant software (version 2.1, AB Sciex). The total MC concentration was calculated as described above.

DNA extraction, 16S rRNA sequencing and sequence processing

DNA extraction of all biofilms from the two experiments was performed using the UltraClean® Water DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's specifications. The purified DNA was eluted in 10 mM Tris buffer and stored at -20 °C. Partial 16S rRNA gene sequences were obtained by Next Generation Sequencing (NGS, Illumina MiSeq V3 subunit (2 x 300bp), LGC Genomics, Germany). The primer pair 799F-1115R was used to amplify DNA of heterotrophic bacteria and exclude chloroplasts[9][65]. The reverse primer was modified by including a degenerate base (5'-AGGGTTGCGCTCGTTRC-3') to increase the coverage against the current SILVA database (version 128) [63]. All samples were analysed in technical replicates. Raw data were then processed by an inhouse pipeline written in DELPHI , which included the joining of paired reads, dereplication, length trimming (250 bases), quality control (0.5 average error rate per sequence), chimera check, and a clustering similar to the UPARSE algorithm [22]. Taxonomic assignment of Operational Taxomonic Units (OTUs) was achieved by a pair-wise alignment (local alignment, Smith-Waterman algorithm, 99 % cut-off similarity) of their representative sequences to the SILVA database.

The two data sets were normalized individually to the lowest total number of reads per sample by an R script [76]. The script rarefied each sample by removing one read from a randomly chosen OTU and repeating this step until the total number of reads in the sample matched the number of reads in the smallest sample. The normalization was performed after OTU calling because OTUs are more robust if calculated on the complete sequence data set. Singletons were excluded after the normalization procedure.

The Bray-Curtis dissimilarity index was calculated using the 'vegan' R package and default R functions (R version 3.3.2) [55] for each pair of samples. Since the technical replicates were not significantly different from each other in this analysis, they were pooled for the subsequent analyses. Significant differences ($P<0.05$) in the read numbers of OTUs between normalized samples were established using the R package 'metagenomeSeq' [59]. Using literature data of the closest cultivated

relatives we then attempted to classify OTUs into genotypes with aerobic or (facultative or obligate) anaerobic metabolism. In total, 53 % of all OTUs representing 91 % reads could be unambiguously assigned to either category. Significant differences across the groups were calculated with Kruskal-Wallis test and Dunn's post-hoc test with Holm's correction, using the 'dunn.test' R package [18] (Table1). For the comparative analysis of the two experiments, a set of 'common OTUs' was defined by only including those OTUs from Exp A and Exp B that were >99.2 % identical (Table 2). Common OTUs represented 60 % of the total number of reads from the normalized dataset of the two experiments. We then produced a data set that only included the reads from the common OTUs and we normalized these data to the sample with the lowest read number as described above. Samples from this subset were clustered using Bray Curtis dissimilarity and average linkage clustering (Figure 5B). A similarity profile analysis ($P<0.001$) identified 15 significantly different groups. We then tested the stability of the 3 major clusters by bootstrapping (1000 bootstrap interactions) using the 'clustsig' [80] and 'fpc' [32] R packages. The same procedure was also carried out on a normalized data set including all OTUs from Exp A and Exp B (Figure 5A).

Results

Experiment A

Physical and chemical parameters

The permeate flux in the LW treatments after the first 24 h period was 13 L m^{-2} h^{-1}. It stabilised after 4 days of operation and remained at around 10 L m^{-2} h^{-1}, resulting in a flux decrease of 23 % (Figure 2A). In the WT and MUT treatments, the permeate flux started at 9.5 L m^{-2} h^{-1} and trended to decrease throughout the experiment without no visible stabilization. Towards the end of the experiment the permeate flux had declined by 82 % in the WT and by 75 % in the MUT treatments. The amended treatments (WT and MUT) had a 75 % lower flux than the LW treatment. The thickness of the biofilms in the various treatments agreed with the respective flux patterns: in that it was <100 µm LW treatment, whereas the WT and MUT biofilms had a thickness in the range of 250 and 1000 µm.

The MC removal efficiency in the WT treatments started at 20 % and reached 100 % over the course of 15 days (Figure 2B). Once the maximum MC removal efficiency was achieved it remained stable until the end of the experiment. The feed water was also monitored in terms of MC concentration and no free toxin was detected.

Microbial community diversity and composition

After normalization and removal of singletons the microbial assemblages in the various treatments of Exp A comprised 2331 OTUs. The WT treatments included 686 and 582 OTUs, the MUT 805 and 909 OTUs and the LW 1569 and 1511 OTUs, respectively to the two biological replicates. All of the treatments had approximately the same number of sequence reads (95000). The LW treatments featured the highest number of exclusive genotypes (Figure 3), with 41 % of the total number of OTUs, whereas substantially lower fractions of OTUs were specific to the WT and MUT treatments, respectively. In terms of reads, the exclusive OTUs from all three treatments together only represented 6.3 % of the total read number. By contrast, 22 % of all OTUs were shared between all treatments (subsequently termed the 'core' assemblage), and these shared genotypes also constituted the vast majority of reads (78 %). The MUT and LW treatments also shared a comparatively high number of OTUs which, however, only represented 3 % of all reads. The WT and LW treatments shared, both the lowest number of OTUs and reads of the three pairs. 147 OTUs representing >10 % of total reads were exclusively present in the WT and MUT treatments. Pairwise comparisons within the core assemblage showed that only 8 OTUs significantly differed in terms of read numbers between the WT and MUT

treatments, whereas there were more than 10 times as many OTUs that differed between the LW and the WT (108 OTUs), or the LW and MUT (120 OTUs) treatments, respectively. In the subset of 147 OTUs that were exclusive to WT and MUT, only 9 significantly differed in read numbers.

Metabolic classification revealed that aerobic genotypes were generally more common, whereas anaerobes dominated in the subset of OTUs that were either exclusive to the WT or that only occurred in both, the WT and MUT treatments (Table 1). The subcommunity shared between the two amended treatments (WT,MUT) featured approximately 30 times higher ratios of anaerobic to aerobic genotypes than all the other shared subcommunities or the core community. In addition, this ratio was >100 times higher for genotypes that were exclusive to the WT treatment then for those that were exclusive to either MUT or LW.

Experiment B

Physical and chemical parameters

The permeate flux in the LW treatment initially ranged around 25 L m^{-2} h^{-1}. It was reduced by 68 % over the following 8 days and stabilised at around 8 L m^{-2} h^{-1} until day 23 (Figure 4A). When MC-containing biomass was added to the LW, the flux decreased compared with the beginning by 87 % during 6 days and stabilised at 1 L m^{-2} h^{-1}. In the WT and MUT treatment the permeate flux after the first 24 h was 5 and 10 L m^{-2} h^{-1}, respectively. In these treatments the flux stabilised after 13 days of operation at 0.5 L m^{-2} h^{-1}, corresponding to a reduction in permeability capacity of around 93 %. The flux in the MUT treatment was not visible affected by the change from MC-free to MC-containing biomass starting on day 23.

MC removal efficiency started at 33 %; it stayed at approximately this level until day 12, then steeply increased and reached 100 % after 20 days (Figure 4B). The MUT treatment, which had been supplied with MC-free biomass until day 23, reached 100 % of MC removal efficiency after only 3 days of exposure to MC-containing biomass. By contrast the LW treatment required 14 more days to also reach maximal MC removal efficiency.

Comparison of microbial community composition between experiments

Altogether, 1054 OTUs were found in the second experiment. Of these, the representative sequences of 614 OTUs were >99.2 % identical to OTUs from Exp A. Cluster analysis on the common genotypes showed that there was a highly significant distinction between the LW treatments from Exp A, the biomass amended treatments (WT & MUT) from Exp A and the three treatments from Exp B

(Figure 5B). Bootstrap analysis further confirmed the stability of these three clusters, and also that the LW treatment of Exp A was significantly separated from the other two clusters. Similarity profile analyses confirmed that the respective technical replicates did not significantly differ from each other. By contrast, such differences were found for the biological replicates, which were, however, consistently more similar within than between treatments. The highest similarity was observed within the LW treatment from Exp B. Cluster and similarity profile analyses of the complete data set of all OTUs from Exp A and B resulted in the same pattern as found for the common OTUs (Figure 5A).

Since microbial community analysis in Exp B was performed after all treatments had been fed with MC-containing cyanobacterial biomass for >2 weeks, we did not further assess the differences between the treatments. Instead, we focused on the subset of OTUs from Exp B that were also present in Exp A. We then selected the 15 common OTUs with the highest read numbers in the common data set, which represent 70 % and 50 % of the total number of reads of the common OTUs and the complete data set of Exp B, respectively (Table 2). Of these top 15 common OTUs, 9 were found to be significantly more abundant in the WT and MUT treatments of Exp A than in the LW, thereby unambiguously qualifying as universal 'responders' to biomass addition across experiments. Moreover, 6 of the top 15 OTUs were closely affiliated (>99 % similarity) to genotypes that are known for their ability to degrade MCs. The majority of these MC-degraders were classified as being aerobic, but two genotype were affiliated with the facultative and obligate anaerobic genera *Aeromonas* and *Acidaminobacter*, respectively.

Discussion

Microbial diversity in GDM biofilms

Microbial biofilm communities are known to express different morphological and phenotypical characteristics than free living cells [74]. Moreover, there seems to be only little genotypic overlap between genotypes that dominate in biofilms and in the water column of freshwater systems. Accordingly, bacterial from the typical planktonic lineages [53] were mostly absent from our GDM biofilms. However, several genotypes in our samples with high read numbers have been previously described as colonizers of suspended organic particles (lake snow) [5], including the two largest OTUs in our 'core' community (i.e., genotypes that were universally present in all treatments), *Ideonella* and *Pelomona*s (Comamonadaceae family). These findings are in agreement with the proposition that biofilms may not only originate from single planktonic cells but also from the attachment of particles and cell aggregates [44]. Other important members of our GDM biofilms belonged to the Comamonadaceae family (*Variovorax* and *Acidovorax*) that are known as prominent components in similar habitats [3][48].

The 'core' community of the GDM biofilms (22 % of OTUs, Figure 3) represented the majority of all reads, implying that a large fraction of the community was shaped by the source rather than selected by the treatments. Previous experiments in a comparable setup [43] also suggested that the core community constituted over 50 % of the total number of reads. Nevertheless, there were clear treatment-specific genotypic shifts in the bacterial assemblages even at low taxonomic resolution: *Alpha-, Betaproteobacteria* and *Bacteriodete*s were represented in similar proportions in the LW treatment, while *Betaproteobacteria* represented almost half of the genotypes in the amended treatments. Pronounced community shifts were also found in stream biofilms upon enrichment with sucrose, nitrogen and phosphorus, but a subset of genotypes were shared between the control and the enriched treatments [35]. Moreover, bacterial diversity decreased in the enriched stream biofilms, which agrees well with our observation from Exp A (Figure 3) [35].

Similarity between WT and MUT treatments in Exp A

Mature biofilms typically develop spatial heterogeneity, which in turn may create vertical niche separation [73][68] In particular, steep oxygen gradients allow for the presence of strict anaerobes in the deeper strata of biofilms that form in fully aerated water [74][4]. In our GDM biofilms, we identified numerous facultative anaerobic bacteria, but also various genotypes from strictly anaerobic lineages, such as *Ignavibacteriales*, *Clostridiales,* or *Desulfovibrionales*. The vast majority (84

%) of all anaerobic reads were found in the WT and MUT treatments, suggesting that the anaerobic lifestyle was a response to a more productive environment. Anaerobic zones and the corresponding genotypes are a common phenomenon in biofilms that develop under substrate-rich conditions in the aerated stages of wastewater treatment plants [54].

Both, the biofilm thickness measurements and the permeate flux (Figure 2A) indicated that the supplementation of biomass was responsible for the morphological and functional properties of biofilms irrespective of toxin content. The two biomass-amended treatments from Exp A, moreover, showed similar ratios of anaerobes to aerobic genotypes (Table 1), likely reflecting a similar physical structuring and community functioning of these biofilms. Genotypic community structure was generally very similar in these two treatments (Figure 3). Moreover, only 8 genotypes were found in the core that were differentially expressed between WT and MUT, and 9 such genotypes in the subset exclusive to the WT and MUT treatments. A previous study on biofilm communities in GDM systems comparing the addition of intact (life) vs. ruptured *M.aeruginosa* cells also found highly similar communities in these treatments that differed from the unamended control [43].

Comparison of the two experiments

One incentive for repeating the experiment at a different season was to test if the addition of cyanobacterial biomass would reproducibly select for a particular microbial sub-community that differed from biofilm communities that were fed with lake water only. However, there are pronounced seasonal differences in the composition of the microbial assemblages of the source water [21]. Thus, the presence of season-specific genotypes might confound any systematic differences between biomass-amended and non-amended biofilms. Therefore, we compared the community structure using the complete data set (Figure 5A), but additionally also only including the genotypes that were common in the two experiments (approximately 22 % of all OTUs or 60 % of all reads).

Genotypic diversity in the two experiments was similar if the fraction of OTUs that were exclusive to the LW treatment in Exp A (962 OTUs) were taken into account (i.e., excluded from the comparison) (Exp A: 1369 OTUs, Exp B: 1054 OTUs). The dissimilarity analysis revealed that the biomass-enriched treatments of the two experiments were clearly separated (Figure 5A). This was also the case even if only common OTUs were considered (Figure 5B), possibly due to the influence of experiment-specific genotypes on their relative proportions (read numbers). At the same time, the most deeply branching separations in both, the complete set of genotypes and in the common OTUs was between the communities from the unamended LW treatment of Exp A and from treatments that had received cyanobacterial biomass. This is evidence that the addition of biomass was the more

383 important factor for determining the community structure of biofilms than the seasonal differences in
384 the composition of the source water.

385 Dissimilarity analysis also confirmed that the technical replicates were not significantly
386 different, indicating that the sequencing effort was sufficient to obtain an unbiased view of individual
387 GDM biofilm communities. Interestingly, this was not the case for biological replicates within identical
388 treatments: While individual biological replicates were always most similar to others from the same
389 treatment, they nevertheless had average Bray-Curtis dissimilarity scores between 40 and 50 % (Figure
390 5). A considerable number of non-singleton OTUs (19 % and 12 % in Exp A and B, respectively) were
391 present in one biological replicate only. The assembly of local communities from a regional species
392 pool is simultaneously driven by stochastic effects such as immigration and drift and deterministic
393 factors, i.e., 'environmental filtering' or 'species sorting' [45][51][47]. High diversity between local
394 assemblages (i.e., beta diversity) might form at identical habitat conditions, e.g., due to priority effects
395 that favour early arriving genotypes. Specifically, higher productivity appears to change community
396 assembly processes towards stochasticity, resulting in greater beta diversity at comparable conditions.
397 [8]. This would be consistent with findings from Exp A: if considering only those genotypes that were
398 specific for a particular treatment type, differences were higher between the various biomass-
399 amended communities than between the ones fed with lake water only (Bray-Curtis dissimilarity
400 indices of 0.78±0.17 and 0.54, respectively). Moreover, Zhou et al demonstrated that even identical
401 environmental conditions can lead to the formation of microbial communities with distinct genotypic
402 composition that all originate from a single inoculum; these communities moreover differed in
403 functional properties [84].

404

405 **'Priming' of biofilm communities for a future function**

406 The striking similarities between the microbial communities in the two biomass-amended
407 treatments from Exp A inspired us to design and perform a second experiment, to explore the notion
408 that GDM biofilm assemblages could be prepared or 'primed' for toxin degradation even without
409 previous exposure to high MC levels. We hypothesised that upon addition of toxic cyanobacteria a
410 mature biofilm pre-fed with non-toxic biomass would achieve complete removal of MC faster than a
411 corresponding biofilm that had formed on lake water only.

412 The outcome of Exp B supported our hypothesis, as the MUT treatment only required 3 days
413 to achieve 100 % of MC removal efficiency after being supplemented with toxic biomass (Figure 4B).
414 By contrast, the same level of performance was only reached after 14 days by the biofilms of the LW
415 treatment. Biofilms from both treatments had been previously grown for a period of two weeks,

suggesting that biofilm 'maturation' in itself was not the key factor for the establishment of (potentially slowly growing) MC degraders, but rather the specific growth conditions induced by the additional biomass. The 10 times higher ratios of anaerobic to aerobic genotypes in the MUT than in the LW treatment (Exp A, Table 1) also suggest that the overall growth conditions in these biofilms were significantly altered, providing an appropriate environment for the establishment of both aerobic and anaerobic MC degraders (Table 2).

The addition of non-toxic biomass was thus sufficient for the MUT treatments to develop the potential for a significantly more rapid degradation of MCs. We propose that this 'cryptic' feature might fall in the category of so-called 'priming' effects [29], i.e., a facilitation of the degradation of recalcitrant compounds by the concomitant availability of readily assimilable organic substrates or nutrients. Currently, there are only few studies that explore the potential of such priming in aquatic systems. Carlson et al. performed incubation experiments with water from two different stations in the Sargasso Sea after addition of surplus carbon, nitrogen and/or phosphorus [7]. At the end of the incubations the total organic carbon was lowest in treatments that had been amended with all 3 substrates and nutrients. Since the overall carbon balance in these treatments was negative the authors interpreted their findings in terms of a 'priming' effect. Our system clearly differs from the above described, as the degradation of MC probably was not directly stimulated by the presence of other, labile organic compounds. Instead, the rich conditions in the MUT treatments likely 'primed' the prior growth of facultative MC degraders that were well-prepared to perform this function once exposed to the toxin. We thus propose to expand the concept of 'priming' from a short-term, ecophysiological perspective to one that also encompasses historical aspects of community composition, i.e., the establishment of microbial populations that are relevant for a specific future function (e.g., toxin degradation) as a consequence of the previously prevailing growth conditions.

The potential for the degradation of xenobiotica is a known 'cryptic' feature of soil microbial communities: Johnsen & Karlson studied the degradation capacity for polycyclic aromatic hydrocarbons (PAHs) by bacteria from different soils, ranging from pristine to heavily contaminated [40]. Pristine soils that were exposed to low background concentrations of PAHs were as much able to degrade the PAHs as the contaminated ones, supporting the idea that bacterial communities might maintain populations capable of metabolizing such compounds even when they are only present in trace amounts. The community scenario of that study differs from ours in that the GDM biofilm assemblages were only formed during the experiment itself. Moreover, the differences in MC degradation between the MUT and LW treatments speaks against the low background concentrations of toxin in the source water (as deduced from the presence of MC-containing cyanobacteria in Lake Zurich [62]) acting as a trigger for the 'priming' effect. Instead, our results clearly indicated that MC

450 degradation in the GDM biofilms was a facultative feature of populations with greater metabolic
451 flexibility that preferably established at richer growth conditions. Known facultative MC degraders
452 include *Pseudomonas aeruginosa* [75] and *Burkholderia sp.* [46].

453

454 **Responders to season and biomass; potential MC degraders**

455 Thirteen of the top 15 common genotypes of the two experiments were significantly enriched
456 in (or exclusive to) the biomass-amended treatments of Exp A (Table 2). Several of these taxa are
457 known to occur in engineered aquatic environments, e.g., *Undibacterium spp.* [42] are typical in
458 drinking water biofilms, and *Zoogloea* [78] and members of the *Blvii28 wastewater-sludge group* [69]
459 are found in waste water treatment systems. Five of the genotypes in Table 2 were represented by
460 very low read numbers in Exp A (<0.1 %), and their respective proportions increased by >10 fold in all
461 treatments of Exp B. These genotypes might be regarded as seasonality-specific responders to biomass
462 and/or MC addition. Three more genotypes also substantially increased in relative abundances in Exp
463 B compared to Exp A, where they only formed between 0.3 and 0.9 % of the reads in the biomass-
464 amended communities, and significantly less in the LW treatment. While this may still hint at seasonal
465 effects, it might equally likely just reflect the intrinsic variability of community assembly between
466 independent experiments, or the at best semi-quantitative nature of community analysis based on PCR
467 amplification of rRNA sequences. Finally, 4 genotypes in Table 2 had high read numbers in both
468 experiments, and at the same time they were significantly less frequent if no cyanobacterial biomass
469 was added (Exp A, LW treatment). Thus they specifically responded to the treatment irrespective of
470 the season.

471 In the absence of additional information (e.g., from pure cultures [57], stable isotope or
472 metagenomic analysis [50], it is not possible to unambiguously identify those bacterial populations
473 that were responsible for MC degradation in the GDM biofilms. Nevertheless, the comparison of the
474 common genotypes from Exp A and Exp B with literature data of known MC-degraders may permit to
475 further narrow down the list of candidates that might have been responsible for this function.
476 Unfortunately, those genotypes that have not yet been marked as MC degraders will be missed by
477 such an approach. Moreover, microbial communities in natural and engineered environments are
478 often characterized by a high degree of functional redundancy [1]. It is, thus, conceivable that an
479 overlapping set of taxa were responsible for MC degradation in the two experiments, i.e., that such
480 bacteria were found within both subsets of genotypes that were either seasonally or constitutively
481 favoured by the addition of cyanobacterial biomass. This is also suggested by the available literature
482 data: Close relatives of six of the 15 most common genotypes in Table 2 are known MC degraders. The

two genotypes with highest read numbers in Table 2 were related to *Aeromonas hydrophila* (only abundant in Exp 2), a common facultative anaerobe in aquatic habitats [14] and *Variovorax paradoxus* (abundant in both experiments), an aerobe found in both, soil and aquatic systems [67]. Both species are known degraders of various complex compounds [58][72] including cyanotoxins: *Aeromonas* spp. were recently reported to degrade MC-LR [49] and cylindrospermopsin [20], and metagenomics information from Lake Erie indicated a potential for MC degradation in *Variovorax spp.*[50]. Other genera in Table 2 with known MC degraders were *Pseudoxanthomonas* [77], *Pseudomonas* [24], *Flavobacterium* [71] and *Acidoaminobacter* [39]. It is also noteworthy that 2 of the genera in Table 2 that harbour MC degraders feature a facultative (*Aeromonas)* or obligate (*Acidoaminobacter)* anaerobic metabolism. Little is known about the biochemistry of anaerobic MCs degradation, even though a number of microbial strains capable of this metabolism have been described, e.g. *Clostridium* [83], *Microbacterium* and *Rhizobium* [64], *Aeromonas* [49] and *Acidoaminobacter* [39]. In addition, there is growing field evidence that this process may be relevant in the sediments of lakes affected by *Microcystis* blooms [82] [34]. In view of the strong indication of anoxic niches in the biomass-amended biofilms (i.e., the proportional increase of reads and genotypes with anaerobic metabolism, Table 1) it is conceivable that the degradation of MCs in our GDM biofilms was performed in parallel by subcommunities of aerobic and anaerobic bacteria.

In summary, our study adds another aspect to our understanding of the beneficial properties of biofilms on the quality of filtrates from GDM systems [61] [43]. Our results suggest that the GDM biofilm communities may develop a potential for the degradation of cyanotoxins even from a prior exposure to a bloom of non-toxic cyanobacteria. This may provide an 'insurance' against MC accumulation in the filtrates if GDM systems are used for drinking water production from sources with infrequent blooms of MC-containing phytoplankton. It remains to be studied if other types of biomass (e.g., blooms of other algal species) would be sufficient to induce a comparable 'priming' of GDM biofilm communities for MC degradation.

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Legends to figures:

Figure 1: Experimental setup of GDM filtration systems. UF: Ultrafiltration

Figure 2: Experiment A: (A) Development of mean permeate flux. (B) Microcystin removal efficiency over 20 days of operation. The two biological replicates of each treatment are represented by black and white symbols and the mean by the solid line. Treatments: WT: Addition of biomass from toxin-containing (wildtype) *Microcystis* sp; MUT: Addition of biomass from toxin-free (mutant) *Microcystis* sp.; LW: fed with lake water without additional biomass

Figure 3: Comparison of the microbial assemblages in the three treatments of Experiment A as derived from 16S rRNA gene defined operational taxonomic units (OTUs). The left and right sets of Venn diagrams represent the proportions of shared OTUs and of shared sequence reads, respectively.

Figure 4: Experiment B: (A) Development of permeate flux in the different treatments (mean ±1 SE, n=3). (B) Microcystin removal efficiency over 39 days of GDM operation. The vertical line at day 23 represents the day when all treatments started to receive MC-containing biomass.

Figure 5: Comparative analysis of Experiments A and B (Bray Curtis dissimilarity), including (A) all genotypes and (B) only those genotypes that were present in both experiments. Similarity profile analysis identified 15 clusters that were significant different from each other (solid lines, $P<0.001$). No significant difference were found between technical replicates (dashed blue line). The stability of the three most deeply branching (i.e., most highly significant) clusters was tested by bootstrapping (indicated by the values at the branching points).

780 **Table 1:** Exp A: Ratios between genotypes (OTUs) that could be unambiguously classified as
781 anaerobes or aerobes, respectively (using either OTU numbers or the number of reads represented
782 by these OTU). The listing distinguishes between genotypes that were exclusively found in one
783 treatment or shared between 2 or 3 treatments. Only mean values are given for the ratios of OTUs
784 that were exclusive to a single treatment (N=2). For the other (shared) OTUs, all possible pairwise
785 combinations of biological replicates were considered for calculating the anaerobe/aerobe ratios
786 (N=4 and 12 for 2 and 3 treatments, respectively). Means and standard deviations of these
787 combinations are given. Differences were assessed for significance by Kruskal-Wallis ANOVAs
788 followed by Dunn's posthoc tests.

789

	Occurrence	Anaerobic/Aerobic	% of total OTUs/reads (Anaerobic)	% of total OTUs/reads (Aerobic)
OTUs	**WT only**	1.5	46	30
reads		16.8	90	5
OTUs	**MUT only**	0.2	10	60
reads		0.2	1	60
OTUs	**LW only**	0.08	3	40
reads		0.03	2	70
OTUs	**WT,MUT**	0.6 (±0.08)	20 (±3)	40 (±4)
reads		4.1 (±1.65)	70(±11)	20 (±7)
OTUs	**WT, LW**	0.1 (±0.06)*	7 (±2)*	60 (±23)
reads		0.1 (±0.11)*	7 (±5)**	60 (±13)
OTUs	**MUT,LW**	0.1 (±0.02)	7 (±1)*	50 (±1)
reads		0.1 (±0.04)*	9 (±2)*	70 (±5)
OTUs	**WT,MUT,LW**	0.1 (±0.01)**	7 (±1)*	60 (±4)**
reads		0.1 (±0.06)*	10 (±5)*	80 (±5)***

790 *P<0.05; **P<0.01; ***P<0.001

791

Table 2: List of 15 OTUs with highest read numbers in Exp B that were >99.2 % similar to OTUs in Exp A. Except for the OTUs marked with asterisks, these OTUs were present in all treatments of Exp A. The fourth column indicates if the respective genotype had significantly higher read numbers in the WT and/or MUT treatments than in the LW treatment of Exp A. (1) Exclusively present in WT and MUT treatments of Exp A. (2) Exclusively present in WT treatment of Exp A.

AccNr (% identity)	Relative Abundance (Exp B, % of common OTUs)	Relative Abundance (Exp A, % of common OTUs)	Δ relative abundance to LW (Exp A)	Related to known MC degraders	Metabolism	Taxonomy (% identity to the closest cultured relative)
AB473022 (100%)	19.5	<0.1	MUT	[49]	Facultative	*Aeromonas hydrophila* (99%)
JQ855508 (99,6%)	7.1	5.5	WT, MUT	[50]	Aerobic	*Variovorax*- to uncultured bacterium (*Variovorax paradoxus* 99%)
AF236013 (100%)	6.5	0.9	WT, MUT		Aerobic	*Undibacterium*-beta proteobacterium A1020 (*Undibacterium terreum* 96%)
AB576897 (100%)	6.0	0.3	MUT	[77]	Aerobic	*Pseudoxanthomonas*-uncultured bacterium (*Pseudoxanthomonas mexicana* 97%)
FM886906 (100%)	4.8	3.5	WT, MUT		Aerobic	*Mitsuaria chitosanitabida* (97%)
AB680727* (100%)	3.9	0.2	(1)		Aerobic	*Flavobacterium xanthum* (100%)
AY212651 (100%)	3.3	0.2	-	[24]	Aerobic	*Pseudomonas*-uncultured bacterium (*Pseudomonas benzenivorans* 97%)
HM141824 (100%)	3.0	1.9	WT, MUT		Anaerobic	Blvii28 wastewater-sludge group-uncultured bacterium (*Parabacteroides distasonis* 83%)
DQ676400* (100%)	2.7	<0.1	(1)		NA	*Rhodocyclaceae*;uncultured-uncultured beta proteobacterium (*Ferribacterium limneticum* 96%)
AB672263 (100%)	2.6	3.6	WT, MUT		Aerobic	*Ideonella*-uncultured bacterium (*Ideonella dechloratans* 98%)
GU454906* (100%)	2.3	<0.1	(2)		Anaerobic	*Spirochaetaceae*;uncultured-uncultured bacterium

						(*Treponema stenostreptum* 90%)
AY212694 (100%)	2.2	0.1	-		Aerobic	*Aquabacterium*-uncultured bacterium (*Aquabacterium commune* 98%)
DQ469224* (100%)	2.2	<0.1	(1)	[71]	Aerobic	*Flavobacterium*-uncultured bacterium (*Flavobacterium succinicans* 99%)
AB672298 (100%)	1.9	<0.1	WT		Aerobic	*Zoogloea*-uncultured bacterium (*Zoogloea resiniphila* 97%)
JX262570 (99,6%)	1.6	0.6	WT, MUT	[39]	Anaerobic	*Acidaminobacter*-uncultured bacterium (*Acidaminobacter hydrogenoformans* 92%)

797
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799
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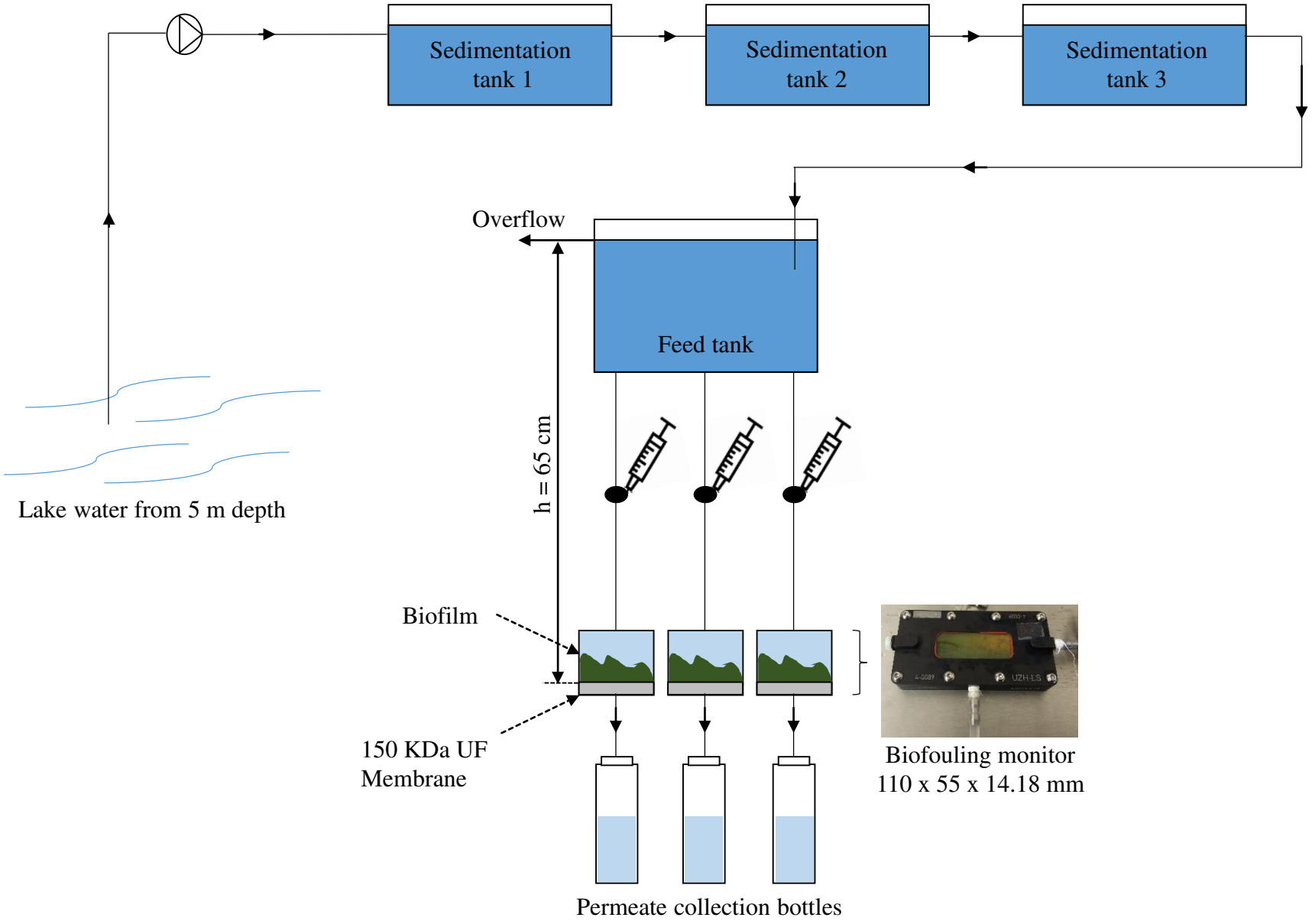
Sedimentation tank 1
Sedimentation tank 2
Sedimentation tank 3
Overflow
Feed tank
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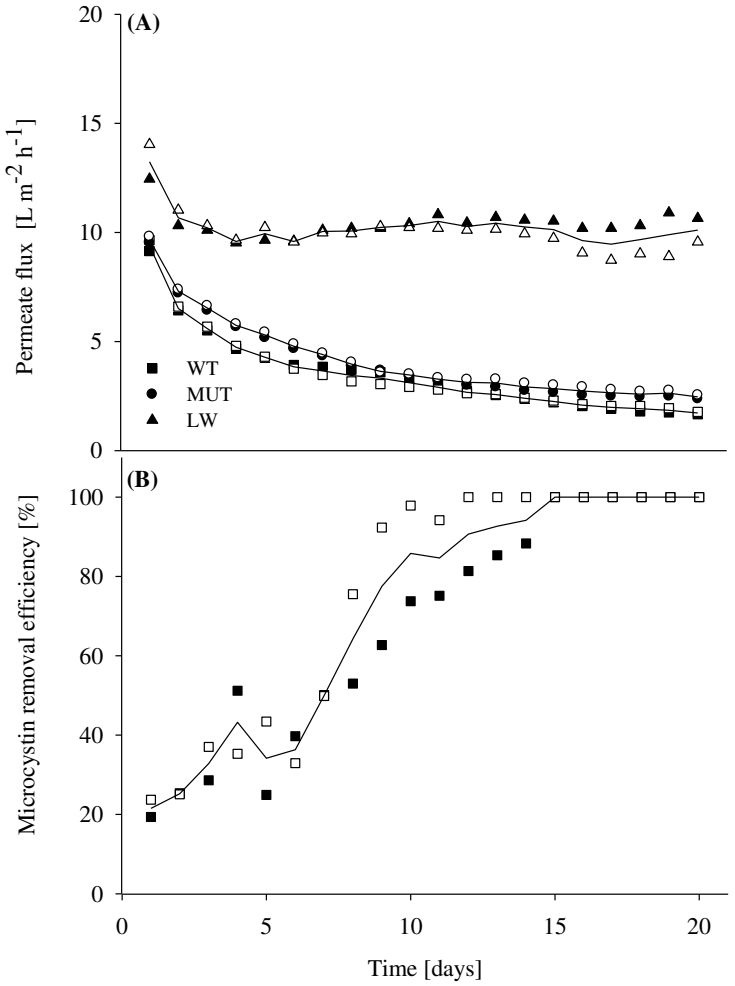
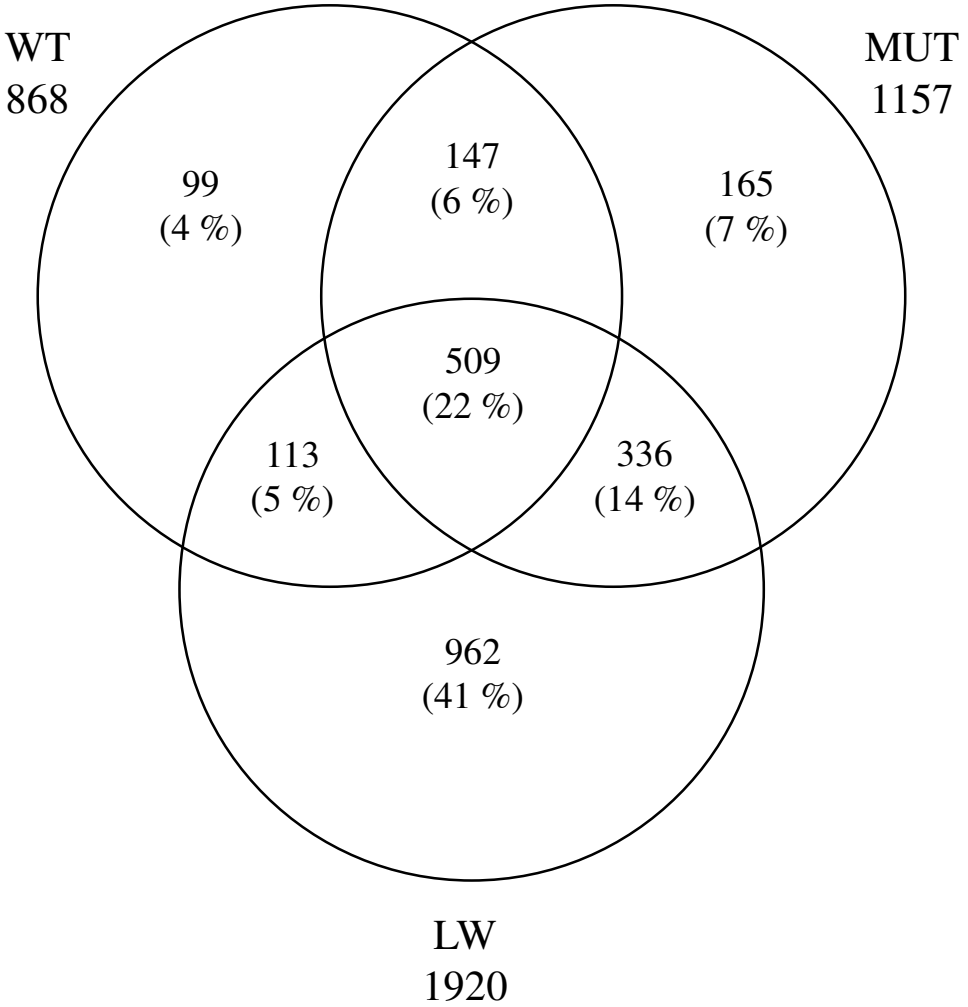
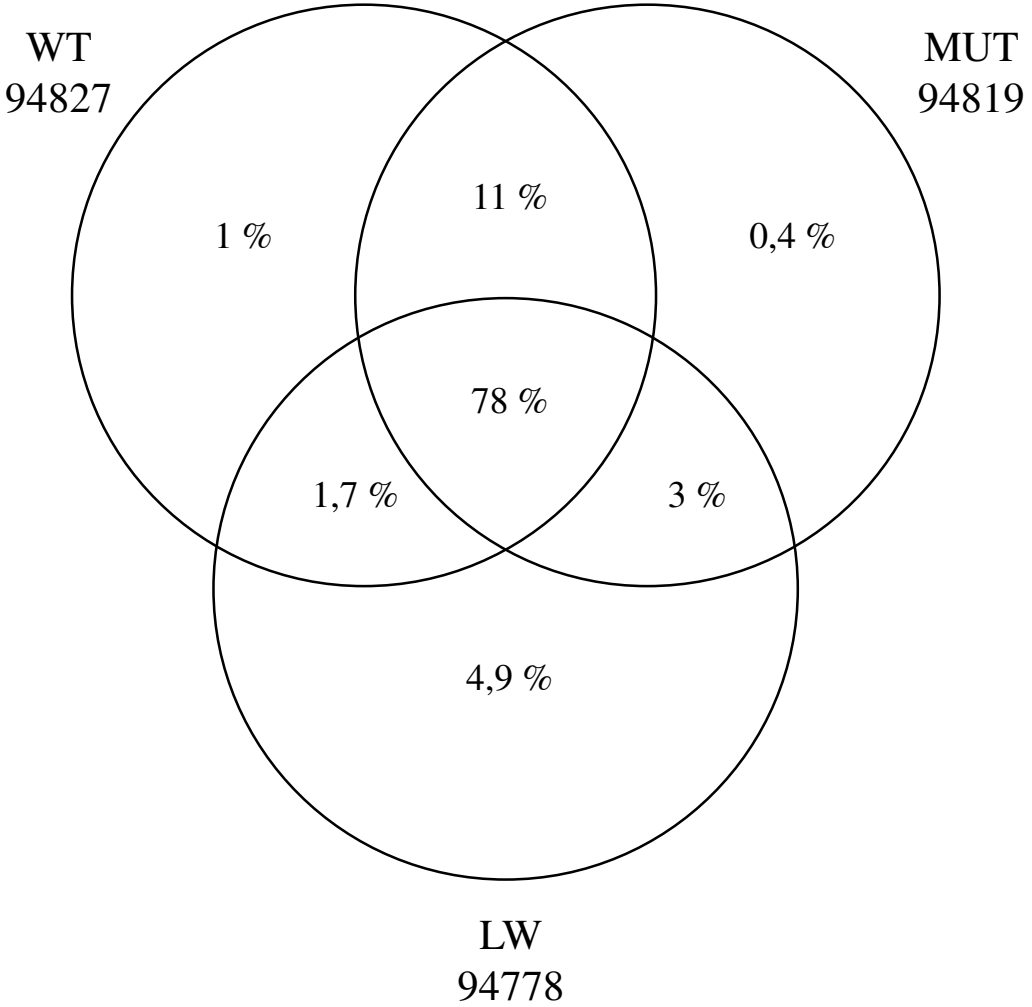
Lake water from 5 m depth
Biofilm
150 KDa UF Membrane
Permeate collection bottles
Biofouling monitor
110 x 55 x 14.18 mm

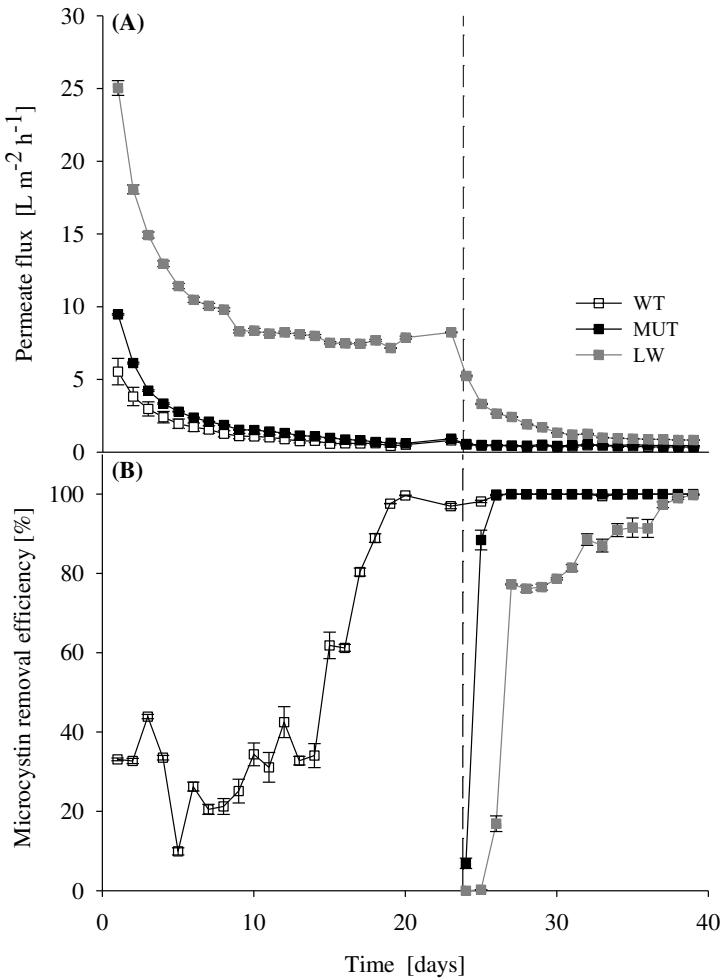

(A)
Permeate flux [L m-2 h-1]
WT
MUT
LW
(B)
Microcystin removal efficiency [%]
Time [days]

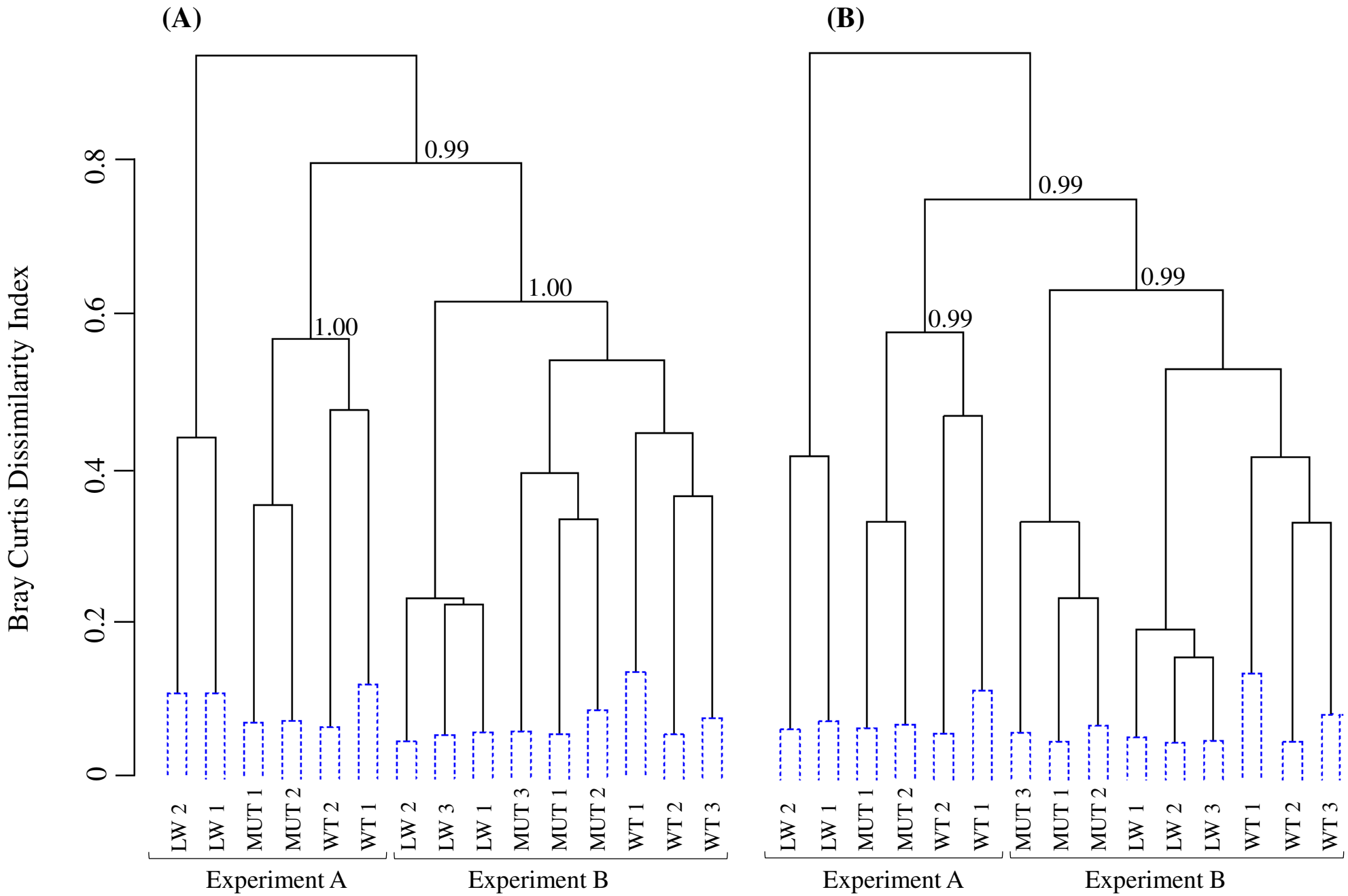
2331 OTUs



284424 reads




(A)
(B)
Permeate flux [L m-2 h-1]
Microcystin removal efficiency [%]
Time [days]
WT
MUT
LW
0
5
10
15
20
25
30
0
20
40
60
80
100
0
10
20
30
40


(A)
(B)
Bray Curtis Dissimilarity Index
0
0.2
0.4
0.6
0.8
0.99
1.00
1.00
0.99
0.99
0.99
LW 2
LW 1
MUT 1
MUT 2
WT 2
WT 1
LW 2
LW 3
LW 1
MUT 3
MUT 1
MUT 2
WT 1
WT 2
WT 3
Experiment A
Experiment B
LW 2
LW 1
MUT 1
MUT 2
WT 2
WT 1
MUT 3
MUT 1
MUT 2
LW 1
LW 2
LW 3
WT 1
WT 2
WT 3
Experiment A
Experiment B